Protein transport into peroxisomes: knowns and unknowns

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Keywords: Peroxisomes, Peroxisomal import machinery, Protein translocation, Protein import, PEX5, PEX7.

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Abstract

Peroxisomal matrix proteins are synthesized on cytosolic ribosomes and rapidly transported into the organelle by a complex machinery. The data gathered in recent years suggest that this machinery operates through a syringe-like mechanism, in which the shuttling receptor PEX5 - the "plunger" - pushes a newly synthesized protein all the way through a peroxisomal transmembrane protein complex - the "barrel" - into the matrix of the organelle. Notably, insertion of cargo-loaded receptor into the "barrel" is an ATP-independent process, whereas extraction of the receptor back into the cytosol requires its monoubiquitination and the action of ATP-dependent mechanoenzymes. Here we review the main data behind this model.

Abbreviations

AAA, ATPases associated with diverse cellular activities; **DTM,** docking/translocation module; **DUB,** deubiquitinating enzyme; **GSH,** glutathione; **PIM,** peroxisomal matrix protein import machinery; **PTS,** peroxisomal targeting sequence; **REM,** receptor export module; **RING,** really interesting new gene; **TPR,** tetratricopeptide repeats.

Introduction

Peroxisomes are single membrane-bound organelles found in almost all eukaryotic organisms [1]. In mammals, they have a relatively simple protein composition, harboring approximately 100 different proteins [2–4]. Despite their structural and functional simplicity, peroxisomes are of vital importance for human health and development, as underlined by a group of genetic diseases, the peroxisomal biogenesis disorders, in which peroxisomes are partially or even completely defective [5, 6]. These disorders are caused by mutations in genes encoding peroxins, proteins specifically involved in peroxisome biogenesis [5–8]. There are 16 such proteins in mammals. Three are involved in peroxisome proliferation (reviewed in [9, 10]). A set of three others is required for peroxisome membrane biogenesis (reviewed in [11, 12]). The remaining 10 peroxins, together with a few additional components, comprise the peroxisomal matrix protein import machinery (PIM), the topic of this essay.

Peroxisomal matrix proteins are transported into the organelle within a few minutes after synthesis in the cytosol [13]. Their correct sorting depends on peroxisomal targeting

sequences (PTSs), small domains in their primary structure that are recognized by shuttling receptors [14–17]. There are two kinds of targeting sequences, the so-called PTS type 1 and 2 (PTS1 and PTS2, respectively; see Fig. 1a for details). PTS1 proteins are transported to the peroxisome by the shuttling receptor PEX5 in all organisms studied up to now [17], whereas PTS2 proteins are delivered to the organelle by a protein complex comprising PEX5 and PEX7 in mammals, plants and many other organisms [14] or PEX7 and a PEX5-like peroxin in yeasts and fungi [14, 18–20] (see Fig. 1a).

PEX5 comprises two major domains (see Fig. 1b). One, encompassing its C-terminal half, consists of two interacting sets of three tetratricopeptide repeats (TPR) which provide the binding site for the PTS1 [21–23]. The other, encompassing its N-terminal half, is an intrinsically disordered domain [24] and harbors a series of short motifs which mediate the interaction of PEX5 with other peroxins [25–27]. In mammals, plants and many other organisms, one of these peroxins is PEX7, a 40-kDa WD-repeat protein that similarly to PEX5 displays a partially cytosolic, partially peroxisomal localization *in vivo* [16, 28]. PEX7 interacts directly with the PTS2 [29–32] and functions as an ancillary factor of PEX5 (or yeasts/fungi PEX5-like peroxins) in the transport of PTS2 proteins to the peroxisome [19, 20, 33, 34].

PEX5 as a holdase-like protein

One of the most remarkable properties of the PIM is its capacity to accept already oligomerized proteins as substrates (reviewed in [35, 36]). Indeed, several studies have shown that when two interacting proteins are co-expressed in the same cell, the existence of a PTS in one of those proteins is sufficient to ensure a peroxisomal localization for at least a fraction of the other protein [37–39]. Those studies led to two conclusions. The first, which remains undisputed and is of major importance to understand the mechanism of the PIM, was that newly synthesized peroxisomal proteins do not have to be unfolded to be translocated across the organelle membrane [37–39]. The second was that most if not all peroxisomal proteins are imported into the organelle only after oligomerization in the cytosol [35, 36], a generalization that is probably incorrect. Indeed, a growing number of observations suggest that many peroxisomal matrix proteins that are oligomeric in their native state actually arrive at the organelle matrix as monomers (reviewed in [40]). The reason for this does not seem to be simply a kinetic property of the protein transport

system, with import of newly synthesized proteins occurring faster than their oligomerization in the cytosol. Rather, recent data suggest that there is an active mechanism that maintains newly synthesized proteins in a monomeric state in the cytosol. Indeed, it was found that PEX5 binds the monomeric versions of several peroxisomal enzymes strongly inhibiting their oligomerization [40–42]. This, together with the fact that the cytosolic PEX5 concentration is probably large enough to bind all newly synthesized proteins that are *en route* to the organelle matrix, led to the proposal that PEX5 is also a chaperone keeping peroxisomal proteins in a near-native monomeric conformation thus blocking premature or unspecific interactions [42].

Interestingly, an analysis of the PEX5-catalase interaction revealed that the N-terminal half of PEX5 is required for its holdase-like activity [42]. Actually, this domain alone inhibits catalase oligomerization, albeit less potently than the full-length protein. This, together with data showing that the N-terminal half of PEX5 from several organisms interacts with at least some cargo proteins [43, 44] suggests that this domain of PEX5 enfolds the cargo protein, thus shielding it from other proteins. It is also possible that a segment(s) of the intrinsically disordered N-terminal half of PEX5 acts as an entropic bristle [45], excluding other proteins from the vicinity of the cargo protein with which it interacts.

Activating the "plunger" – the auto-regulatory mechanism of PEX5

As described below, PEX5 becomes transiently inserted into a peroxisomal transmembrane protein complex – the Docking/Translocation Module (DTM) – at a certain stage of the protein transport cycle. Although none of the steps leading to that stage require energy from NTP hydrolysis, the subsequent extraction of PEX5 from the DTM does consume ATP [46]. Thus, it is not surprising that the PIM avoids futile energy-costing cycles by ensuring that only PEX5 molecules carrying a cargo protein have access to the DTM [47, 48]. Interestingly, this regulatory mechanism resides not in the DTM but rather in PEX5 itself. Indeed, only intact PEX5 molecules are impeded from entering the DTM in the absence of cargo proteins; truncated PEX5 species lacking the PTS1-binding domain no longer display this property, that is, they enter the DTM in an unregulated, constitutive manner [47]. Apparently, the C-terminal half of PEX5 is a cis-acting repressor of PEX5 DTM-interacting domains, which reside in its N-terminal half.

Although structural information on the auto-regulatory mechanism of PEX5 is presently unavailable, it is possible that it relies on intramolecular interactions involving these two domains of PEX5 (see Fig. 1c). Several findings support this possibility. First, pull-down assays using recombinant proteins have shown that the two halves of PEX5 can interact with each other [49]. Second, the conformation of the N-terminal half of PEX5 is altered when PEX5 binds a PTS1 peptide as assessed by partial proteolysis experiments [50, 51]. Finally, a single missense mutation in the PTS1-binding domain of PEX5 that abolishes its PTS1-binding capacity induces conformational alterations in the N-terminal half of PEX5 and, importantly, also disrupts its auto-regulatory mechanism [50].

Assembling the syringe – interaction of cargo-loaded receptors with the DTM

The DTM (the syringe barrel) comprises 5 core components: PEX14, PEX13 and the three RING finger proteins PEX2, PEX10 and PEX12 [52, 53]. Despite an abundance of information on binary interactions between DTM components [26, 52–62], the precise organization of these proteins in the peroxisomal membrane is not known. The fact that this protein complex largely falls apart upon solubilization of the peroxisomal membrane has complicated its structural characterization [52, 53]. Nevertheless, it is clear that all of its components are transmembrane proteins, and that at least two of them, PEX14 and PEX13, have the capacity to homoligomerize and to interact directly with PEX5 [25–27, 51–65]. The PEX5-binding domains of PEX13 are exposed into the cytosol whereas the strongest PEX5-binding domain of PEX14 is either deeply embedded in the peroxisomal membrane or even exposed into the peroxisome matrix [64, 66–68]. Thus, PEX14 and PEX13 are probably the major components of the protein translocation channel (see also below).

The interaction of cargo-loaded PEX5 with the DTM occurs in two steps: docking and insertion (see Fig. 2). The first is a reversible step whereas the second is essentially irreversible in the absence of ATP [46, 69, 70]. Remarkably, DTM-inserted PEX5 displays a transmembrane topology, exposing only a small N-terminal domain into the cytosol whereas the bulky part of its polypeptide chain faces the organelle matrix [71]. Such a topology suggests that cargoes are translocated across the peroxisomal membrane by PEX5 itself, when the receptor gets inserted into the DTM [71]. Importantly, insertion of PEX5 into the DTM does not require NTP hydrolysis *in vitro* and, accordingly, the

same was recently shown to be the case for the peroxisomal import of both PTS1 and PTS2 proteins [46, 69, 72]. These findings, together with data showing that protein import into peroxisomes is a ionophore-insensitive process [72–74], strongly suggest that the driving force for protein translocation across the peroxisome membrane resides in simple protein-protein interactions involving PEX5, on one side, and components of the DTM, on the other [46, 69, 72].

The finding that PEX5, an extremely hydrophilic protein lacking any obvious phylogenetically conserved membrane-interacting domains, acquires a transmembrane topology during the cargo protein translocation step, together with the fact that the peroxisomal membrane is impermeable to all but the smallest of the metabolites [75], also provides some information on the architecture of the DTM. Indeed, it suggests that DTM components form a flexible and gated channel in which cargo-loaded PEX5 becomes inserted to release its cargo into the peroxisome matrix. We note, however, that there are other perspectives. An interesting one can be found in the so-called transient pore model [76]. According to that model, protein translocation across the peroxisomal membrane is promoted by one or even several PEX5 molecules all of which become inserted into the peroxisomal lipid bilayer thus forming the hydrophilic channel through which cargoes are translocated [76, 77]. Two arguments seem to be at the root of that model. The first derives from the idea that most peroxisomal proteins might be imported into peroxisomes after oligomerization in the cytosol. Since some of these oligomers expose several PTSs on their surface, they might interact with several PEX5 molecules and be presented to the DTM as such [76, 77]. However, as discussed above, peroxisomal import of already oligomerized cargoes may not be that frequent. The second argument regards the fact that once at the peroxisome, PEX5 cannot be extracted from the organelle membrane by treatment with alkaline solutions, a property that might suggest that peroxisomal PEX5 is an intrinsic membrane protein (*i.e.*, that it interacts directly with the hydrophobic phase of the membrane) [23, 78–80]. However, we have recently found that the interaction between PEX5 and PEX14, one of the most abundant components of the DTM, is remarkably stable at alkaline pH even in the absence of membrane lipids (Dias *et al.*, unpublished). Thus, there is no need to assume that peroxisomal PEX5 interacts directly with the hydrophobic phase of the membrane to explain its biochemical properties.

After insertion of cargo-loaded PEX5 or PEX5^{PEX7} into the DTM, cargoes are released into the organelle matrix. Interestingly, and similarly to the insertion step, cargo-release is also a NTP-independent event and insensitive to several ionophores [69, 72]. Possibly, the receptors undergo conformational alterations during the insertion step which decrease their cargo-binding affinity, as is in fact supported by protein-protein interaction studies suggesting that some receptor-cargo interactions are decreased or even abolished by PEX14 [42, 81, 82].

The receptor recycling machinery

After cargo-release the shuttling receptors are extracted from the DTM so that they can engage in a new protein transport cycle. This is the only segment of the protein import pathway that requires energy input from ATP hydrolysis [46]. Thus, in contrast to many other protein import machineries, which use ATP/GTP hydrolysis as the driving force for the vectorial translocation of proteins across a membrane [83, 84], the PIM uses ATP not for the protein transport process itself, but rather to reset the protein transport system.

The machinery involved in receptor recycling comprises at least 10 proteins. Three of these are the DTM RING peroxins, PEX2, PEX10 and PEX12. They form a subcomplex within the DTM, as shown by biochemical and genetic studies in yeasts [53, 85]. Also, the RING domains of all three peroxins display ubiquitin-ligase activity in *in vitro* assays [86–89].

Three other components of this machinery are PEX1, PEX6, and a poorly conserved tail-anchored peroxin of the peroxisome membrane called PEX26 in mammals and many other organisms, APEM9 in plants and PEX15 in yeasts and some fungi [18, 90–94]. These peroxins comprise the so-called Receptor Export Module (REM), a protein complex that uses ATP hydrolysis to extract receptors from the DTM [46, 90, 91]. PEX1 and PEX6 are members of the AAA family of mechanoenzymes; they form a heterohexameric ring, best described as a trimer of PEX1/PEX6 heterodimers, which is anchored to the peroxisome membrane by PEX26/APEM9/PEX15 [92–98]. Finally, the export machinery also comprises ubiquitin, an ubiquitin-activating enzyme, an ubiquitin-conjugating enzyme (E2D1/2/3, in mammals; the PEX4-PEX22 complex in yeasts, fungi and plants), and AWP1, a proposed ubiquitin-binding adaptor of the mammalian PEX1/PEX6 complex [99–101].

Disassembling the syringe – recycling of receptors

Extraction of PEX5 or PEX5^{PEX7} from the DTM involves two distinct steps, namely, monoubiquitination of PEX5 and ATP-dependent dislocation of monoubiquitinated PEX5 (Ub-PEX5) back into the cytosol. PEX7 is not ubiquitinated during this event but its export requires monoubiquitination and dislocation of PEX5 from the DTM. Interestingly, Ub-PEX5 and PEX7 seem to leave the DTM separately, with the former displaying faster export kinetics than the latter. Apparently, extraction of Ub-PEX5 from the DTM also disrupts its interaction with PEX7. How exactly PEX7 is subsequently released into the cytosol is still unknown but it might simply involve the spontaneous disruption of a weak protein-protein interaction with the DTM [48, 102]. Monoubiquitination of PEX5 displays some noteworthy properties. First, in contrast to classical ubiquitination which targets lysine residues, the final acceptor of ubiquitin is a phylogenetically conserved cysteine residue present in the small cytosol-exposed domain of DTM-embedded PEX5 [100, 103] (see Fig. 1b). The reason for this is still not fully comprehended but, as discussed elsewhere [104], this type of unconventional ubiquitination may, on one hand, allow a redox regulation of the PEX5-mediated protein import pathway and, on the other, reduce the probability that dislocated (*i.e.*, cytosolic) Ub-PEX5 ends up in the ubiquitin-proteasome pathway. Data supporting both possibilities have been provided recently [105, 106]. Second, monoubiquitination of PEX5 at the DTM is an integral and mandatory step of the PEX5 peroxisome-cytosol cycle, and not the result of some regulatory event occurring at the PIM [103, 104, 107, 108]. Finally, monoubiquitination of PEX5 is completely dependent on the three RING peroxins and occurs only after cargo-dependent insertion of the receptor into the DTM [48, 69, 109]. The actual monoubiquitination mechanism remains largely uncharacterized. It is known that E2D1/2/3 in mammals and the PEX4-PEX22 complex in yeasts are the ubiquitin conjugating enzymes involved in this step [99, 100] but which of the RING peroxin(s), if any alone, participate in this reaction remains undefined [110].

After monoubiquitination, PEX5 is dislocated by the REM. Recognition of Ub-PEX5 by the REM probably involves a direct interaction between the ubiquitin moiety and the REM because modification of cys11 of DTM-embedded PEX5 with a bulky ubiquitin analog results in a PEX5 protein that is no longer an export substrate [103]. Thus, it is not

monoubiquitination of PEX5 *per se* but rather the protein interface provided by an intact ubiquitin bound to PEX5 that triggers the REM. However, it is unlikely that the Ub-PEX5.REM interaction is limited to this single-site contact. Indeed, some recent data suggest that PEX5 itself and PEX14 may also interact directly with the REM [90, 111].

Extraction of DTM-embedded Ub-PEX5 into the cytosol is very fast *in vitro* (half-life < 2 min) [48, 70]. This step is absolutely dependent on ATP hydrolysis but the stoichiometry of the reaction, *i.e.*, the number of ATPs hydrolyzed *per* Ub-PEX5 dislocated, is unknown. The same is true for the mechanism used by PEX1/PEX6 to extract Ub-PEX5 from the DTM. Although the structure of the yeast complex was recently determined, the data did not unveil its mechanism, as discussed recently [98]. Nevertheless, by analogy with other members of the AAA family it was proposed that Ub-PEX5 is moved "into, and perhaps even through," the central pore of the PEX1/PEX6 ring [98]. A recent finding might favor the second possibility, *i.e.*, that at least a portion of PEX5 polypeptide chain is threaded through the REM pore during the extraction step. Indeed, it has been shown that a PEX5 protein harboring a bulky EGFP moiety at its C-terminus can still enter the DTM, where it is monoubiquitinated and recognized by the REM. However, its export is severely compromised resulting in the accumulation of a partially dislocated species having most of the PEX5 moiety already exposed into the cytosol while the EGFP portion plus a few PEX5 C-terminal residues is still associated with the organelle, presumably trapped at the REM [112].

Closing the cycle – PEX5 deubiquitination

Dislocation of Ub-PEX5 into the cytosol is followed by its rapid deubiquitination, the last step of the PEX5-mediated protein import pathway. The most active deubiquitinating enzyme (DUB) involved in this event has been identified in both yeast and mammals. These are UBP15 and USP9X, respectively [107, 113]. However, it is clear that these enzymes do not provide the only way to deubiquitinate PEX5 because deletion or knock-down of the corresponding genes does not lead to the accumulation of cytosolic Ub-PEX5. Possibly, other DUBs also contribute to this reaction. It is also feasible that a fraction of Ub-PEX5 is deubiquitinated in a non-enzymatic manner since the labile thioester bond linking ubiquitin to PEX5 becomes highly sensitive to nucleophilic attack (*e.g.*, by glutathione (GSH)) after extraction of Ub-PEX5 from the DTM [104].

Conclusions and outlook

Our knowledge on the PEX5-mediated protein import pathway has increased remarkably in recent years. Yet, it is evident that there are still large gaps in our understanding of the PIM. A particularly large one regards the composition and architecture of the hydrophilic channel through which matrix proteins are translocated into the peroxisome matrix. Clearly, we need the power of structural biology to get at least some snapshots of how all the proteins that comprise the DTM are organized. Another line of research that will surely provide valuable information regards the functional/structural characterization of the PIM in more divergent/ancient organisms. For instance, some PEX14 proteins (*e.g.*, GeneBank acc. number: EJK45126.1, [114]) possess a PUB domain, which is known to mediate interactions with p97 [115], a protein that similarly to PEX1/6 is a member of the AAA family of mechanoenzymes. If true, this might suggest that at a certain time in evolution p97 was the mechanoenzyme in charge of dislocating receptors from the DTM. Clearly, we will see many exciting discoveries on the peroxisomal matrix protein import machinery in the coming years.

Competing financial interests

The authors declare that they have no competing financial interests.

Acknowledgments

We would like to thank Dr. Marc Fransen (KU Leuven) for his critical reading of the manuscript. This work was financed by FEDER - Fundo Europeu de Desenvolvimento Regional, funds through the COMPETE 2020 - Operacional Programme for Competitiveness and Internationalization (POCI), Portugal 2020, and by Portuguese funds through FCT - Fundação para a Ciência e a Tecnologia/ Ministério da Ciência, Tecnologia e Inovação in the framework of the projects "Institute for Research and Innovation in Health Sciences" (POCI-01-0145-FEDER-007274) and “The molecular mechanisms of peroxisome biogenesis” (PTDC/BEX-BCM/2311/2014), and through Norte 2020 – Programa Operacional Regional do Norte, under the application of the “Porto Neurosciences and Neurologic Disease Research Initiative at i3S (NORTE-01-0145-FEDER-000008)”. T.F., T.A.R., A.F.D., A.B.B. and D.B. were supported by

Fundação para a Ciência e a Tecnologia, Programa Operacional Potencial Humano do QREN and Fundo Social Europeu.

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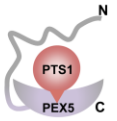
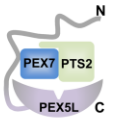
Figures legend

Figure 1. A: The peroxisomal targeting signals. The PTS1 is a small peptide present at the extreme C-termini of many peroxisomal matrix proteins. It frequently ends with the sequence SKL [17]. These proteins are recognized by the shuttling receptor PEX5 [17]. The PTS2 is a degenerated nonapeptide found at the N-termini of a few proteins [14]. In mammals, plants and many other organisms, PTS2 proteins are transported to the peroxisome by a $PEX5^{PEX7}$ protein complex [14], whereas in yeasts and fungi this is done by a complex comprising PEX7 and a PEX5-like peroxin [14, 18, 19]. It is possible that newly synthesized PTS2 proteins interact first with cytosolic PEX7, and that PEX5 (or PEX5-like peroxins) joins the complex subsequently [116, 117]. **B: Functional/structural domains of PEX5.** The N-terminal half of PEX5 is an intrinsically disordered region. It contains eight pentapeptide motifs (shown in dark gray) all of which have been shown to interact with PEX14 [26, 27]. Some of these motifs also interact with PEX13 [26]. The conserved cysteine residue (cysteine 11 in human PEX5), and a PEX7/PTS2-binding region (shown in blue; [19, 20, 29, 102, 117] are also indicated. The structure of the C-terminal half of human PEX5 is known [21] (PDB ID: 1FCH). The TPRs (shown in magenta) form the PTS1-binding domain of PEX5. **C: The auto-regulatory mechanism of PEX5.** The hypothetical model shown is based on the finding that the C-terminal half of PEX5 is a cis-acting repressor of the DTM-interacting domain of PEX5. Free PEX5 oscillates between two conformations, one inactive and the other active, the latter being much less populated than the former; cargoes interact with the active form of PEX5, maintaining the DTM-interacting region of PEX5 in an active state. Transition between inactive and active PEX5 might also be triggered by binding of the cargo protein to PEX5; however, this possibility would not explain why over-expression of PEX5 in cells leads to a partial inhibition of peroxisomal import [118].

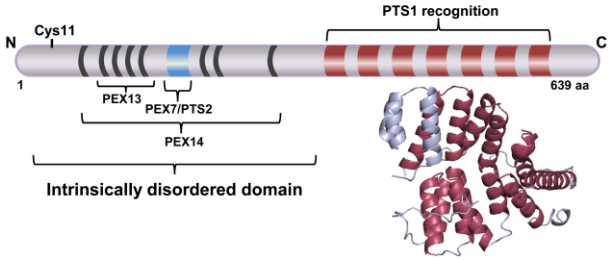
Figure 2. The peroxisomal matrix protein import mechanism. Peroxisomal matrix proteins (CP) are synthesized on cytosolic ribosomes and bound by the shuttling receptor PEX5 (stage 1a). The PEX5-cargo complex then docks at (stage 1b) and becomes inserted into the DTM (stage 2) resulting in cargo translocation across the peroxisomal membrane and its release into the organelle matrix. PEX5 is then monoubiquitinated at a conserved cysteine residue (cysteine 11 in mammals) (stage 3a), a mandatory

modification for the subsequent interaction with the receptor export module (REM; stage 3b). Finally, after the ATP-dependent extraction of monoubiquitinated PEX5 into the cytosol (stage 4), PEX5 is deubiquitinated probably by a combination of non-enzymatic (*e.g.*, glutathione (GSH)) and enzymatic mechanisms (*e.g.*, USP9X in mammals). Free PEX5 (stage 0) can then start a new protein transport cycle. Import of PTS2-containing proteins involves the receptor $PEX5^{PEX7}$. PEX7 remains bound to PEX5 during most of the steps of this pathway (see also [48, 102]). For simplicity PEX7 is not shown in the figure. E1, ubiquitin-activating enzyme, E2, ubiquitin-conjugating enzyme (*i.e.*, E2D1/2/3), E3, ubiquitin RING ligases (*i.e.*, PEX2, PEX10 and PEX12), PPi, pyrophosphate, Ub, ubiquitin.

A)

Peroxisomal targeting sequence	Receptor-cargo complex
PTS1 C-terminal: (S/A/C)-(K/R/L)-(L/M)	
PTS2 N-terminal: R-(L/V/I/Q)-X-X-(L/V/I/H)-(L/S/G/A)-X-(H/Q)-(L/A)	

B)



C)

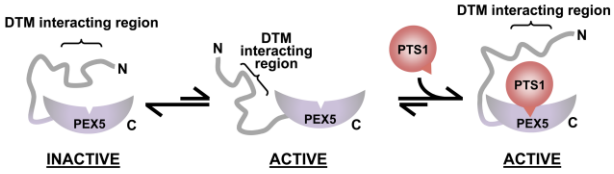


Figure 1

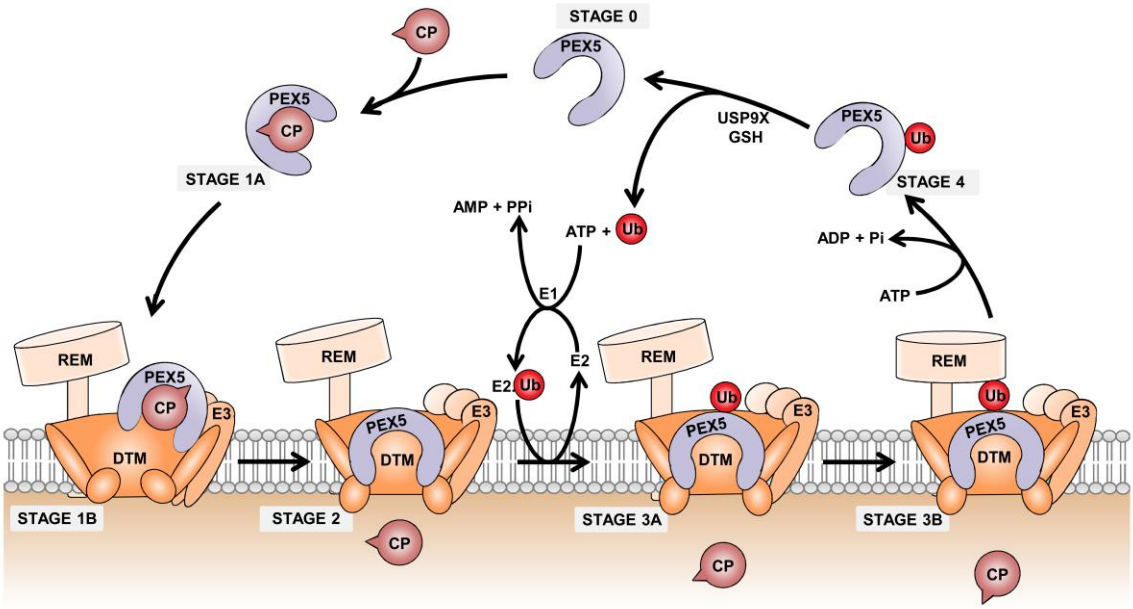

STAGE 0
PEX5
CP
PEX5
CP
STAGE 1A
USP9X
GSH
PEX5
Ub
STAGE 4
AMP + PPi
ATP + Ub
E1
ADP + Pi
ATP
REM
PEX5
CP
E3
DTM
E2
E2 Ub
Ub
STAGE 1B
STAGE 2
STAGE 3A
STAGE 3B
CP
CP
CP

Figure 2